Postnatal maternal separation modifies the response to an obesogenic diet in adulthood in rats

Laura Paternain1, Eva Martisova2, Fermín I. Milagro1, María J. Ramírez2, J. Alfredo Martínez1 and Javier Campión1,*

SUMMARY
An early-life adverse environment has been implicated in the susceptibility to different diseases in adulthood, such as mental disorders, diabetes and obesity. We analyzed the effects of a high-fat sucrose (HFS) diet for 35 days in adult female rats that had experienced 180 minutes daily of maternal separation (MS) during lactancy. Changes in the obesity phenotype, biochemical profile, levels of glucocorticoid metabolism biomarkers, and the expression of different obesity- and glucocorticoid-metabolism-related genes were analyzed in periovaric adipose tissue. HFS intake increased body weight, adiposity and serum leptin levels, whereas MS decreased fat pad masses but only in rats fed an HFS diet. MS reduced insulin resistance markers but only in chow-fed rats. Corticosterone and estradiol serum levels did not change in this experimental model. A multiple gene expression analysis revealed that the expression of adiponutrin (Adpn) was increased owing to MS, and an interaction between HFS diet intake and MS was observed in the mRNA levels of leptin (Lep) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Ppargc1a). These results revealed that early-life stress affects the response to an HFS diet later in life, and that this response can lead to phenotype and transcriptomic changes.

INTRODUCTION
Obesity is defined as a disproportionate accumulation of body fat mass, which is normally accompanied by an excessive increase in body weight, making this chronic disease one of the most serious public health problems around the world (Marti et al., 2008). The rapid increase in the prevalence of obesity is not only attributable to genetic causes (Moleres et al., 2009) or to adult lifestyle factors such as lack of physical exercise and consumption of high fat and high glycomic index diets (Astrup et al., 2008), but also to early-life determinants and epigenetic marks that are increasingly recognized as being of great importance (Cagampang et al., 2011).

The postnatal environment plays a relevant role in neurodevelopment and behavioral responses later in life (Maniam and Morris, 2010). Indeed, several animal studies and human epidemiological data support the notion that fetal programming and neonatal events can have long-term or permanent effects. These become characteristic of the individual (Loizzo et al., 2006), because a stressor acting in critical periods during early life can alter structure, physiology and metabolism, causing permanent dysfunctions (Godfrey and Barker, 2001). Moreover, an adverse early-life environment has been postulated to be involved in the susceptibility to different diseases in adulthood, such as mental disorders, cancer, diabetes mellitus and obesity (Burdge et al., 2009). In this context, maternal separation (MS) is a well-known animal paradigm (Levine, 2002; O’Mahony et al., 2009), resulting in animals with behavioral and neuroendocrine signs of elevated stress reactivity as adults (Aisa et al., 2007; Ladd et al., 2000; Solas et al., 2010).

Furthermore, it has been described that stress can reduce body weight in rodents (Garcia-Diaz et al., 2007; Lin et al., 2005; Marin et al., 2007; Paternain et al., 2011), but there are controversial results in experimental studies combining a hypercaloric diet and stress (Garcia-Diaz et al., 2007; Isingrini et al., 2010; Kamara et al., 1998; Kuo et al., 2008; Kuo et al., 2007; Levin et al., 2000; Pecoraro et al., 2006; Tamashiro et al., 2007). These contrasting outcomes might be caused by the paradigms used, but also might depend on the species and strains or the type and composition of the diet. In this sense, it has been extensively reported that adverse behavioral actions and obesity are related to hypothalamic metabolism (Torres and Nowson, 2007), but little is known about the peripheral processes by which these factors affect adiposity and insulin resistance (Kuo et al., 2007). Based on this background, in the present work we have evaluated the peripheral effects of high-fat sucrose (HFS) diet intake on adult female rats that had experienced MS.

RESULTS
Effects of an HFS diet and MS on body weight gain and other corporal measurements
In adult rats, HFS diet intake induced the expected overweight model and this effect was reflected in a higher body weight gain ($F_{3,33}=49.04$, $P<0.001$; $n=6-11$), final body weight ($F_{3,33}=16.112$, $P<0.001$; $n=6-11$), food intake ($F_{3,33}=12.436$, $P<0.005$; $n=2-3$) and energy efficiency ($F_{3,33}=23.182$, $P<0.001$; $n=6-11$) (Table 1). Regarding adiposity, there were significant increases in visceral fat (calculated as the sum of periovaric, retroperitoneal and mesenteric fat pads; $F_{3,33}=26.279$, $P<0.001$; $n=6-11$) and total fat (calculated as the sum of visceral and subcutaneous fat pads; $F_{3,33}=27.069$, $P<0.001$; $n=6-11$), including all the analyzed depots separately [retroperitoneal white adipose tissue (WAT), $F_{3,33}=50.754$, $P<0.001$; $n=6-11$; subcutaneous WAT, $F_{3,33}=26.861$, $P<0.001$; $n=6-11$; mesenteric WAT, $F_{3,33}=32.222$, $P<0.005$; $n=5-11$; periovaric WAT, $F_{3,33}=...
F3,32=23.803, P<0.01; n=6-10) (Fig. 1). The MS paradigm during lactation reduced overall fat depots in adulthood without modifying food intake (Kcal/day) (Fig. 1B-F) except for the retroperitoneal fat pads. C, control; MS, maternal separation; HFS, high-fat sucrose; HFS-MS, high-fat-sucrose–maternal-separation; ns, not significant.

Effects of an HFS diet and an early-life stress on biochemical overweight biomarkers

Biochemical measurements at the end of the dietary treatment confirmed that the HFS diet induced different abnormalities leading to the common features associated with obesity and metabolic syndrome in rats, such as higher serum leptin levels (F3,32=27.231, P<0.001; n=6-11) and glucose levels (F3,32=8.142, P<0.01; n=6-11), and lower serum triglyceride (F3,32=12.870, P<0.05; n=6-11), cholesterol (F3,32=17.853, P<0.001; n=6-11), high-density lipoprotein (HDL; F3,32=22.114, P<0.001; n=6-11) and free fatty acid (FFA; F3,32=15.479, P<0.001; n=5) levels (Table 2).

With regard to insulin resistance biomarkers, there was an interaction between the dietary treatment and MS paradigm in serum insulin levels (F3,30=9.376, P<0.01; n=5-11), as well as in the homeostasis model assessment (HOMA) index (F3,30=6.685, P<0.05; n=5-11). Further analysis revealed a significant decrease in insulin resistance markers in chow-fed rats only. Finally, neither HFS diet intake nor MS protocol induced significant changes during fasting state on serum corticosterone levels and estradiol levels, measured as biomarkers of the hypothalamic adrenocortical axis, or in serum MCP-1 levels, assessed as an inflammation marker.

Effects of an HFS diet and MS on gene expression in periovaric fat

The analysis using a fold-change cut-off of 1.5 of the reverse-transcriptase PCR (RT-PCR) array in a small sample (n=20) showed up to 9 of 52 mRNA values as relevant in the study (supplementary material Table S1). However, after the validation of these 9 genes material Table S1). However, after the validation of these 9 genes material Table S1), up to 3 of 9 mRNA values were considered as differentially expressed due to an HFS diet, MS or both together (Table 3). Thus, the HFS diet in rats over 5 weeks induced an increase of Ppargc1a (F3,30=16.678, P<0.001; n=6-11)

Table 1. In vivo and food-intake measurements, and statistical analysis of the four groups

<table>
<thead>
<tr>
<th>Measurement</th>
<th>C (n=6)</th>
<th>MS (n=10)</th>
<th>HFS (n=7)</th>
<th>HFS-MS (n=11)</th>
<th>Diet</th>
<th>MS</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy efficiency (%)</td>
<td>1.2±0.19</td>
<td>1.2±0.12</td>
<td>2.1±0.11</td>
<td>1.9±0.20</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Food intake (Kcal/day)</td>
<td>57.4±0.18</td>
<td>56.6±0.73</td>
<td>60.8±2.35</td>
<td>61.9±1.12</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>25.7±3.41</td>
<td>23.8±1.85</td>
<td>45.9±2.96</td>
<td>44.4±3.02</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>220.2±6.24</td>
<td>221.4±3.61</td>
<td>239.3±6.07</td>
<td>243.5±4.72</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Initial weight (g)</td>
<td>194.5±3.86</td>
<td>197.6±2.81</td>
<td>193.4±4.33</td>
<td>199.2±3.08</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Results are expressed by mean ± s.e.m. C, control; MS, maternal separation; HFS, high-fat sucrose; HFS-MS, high-fat-sucrose–maternal-separation; ns, not significant.
Table 2. Biochemical measurements and statistical analysis of the four groups

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control (n=6)</th>
<th>MS (n=10)</th>
<th>HFS (n=7)</th>
<th>HFS-MS (n=11)</th>
<th>Diet</th>
<th>MS</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.2±0.25</td>
<td>5.2±0.18</td>
<td>5.8±0.23</td>
<td>5.9±0.21</td>
<td>&lt;0.01</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>52.1±4.81</td>
<td>44.3±2.88</td>
<td>37.6±2.27</td>
<td>37.3±2.08</td>
<td>&lt;0.05</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>73.7±5.54</td>
<td>76.6±4.77</td>
<td>56.4±3.45</td>
<td>59.0±2.37</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>26.8±1.51</td>
<td>26.8±1.24</td>
<td>21.3±1.14</td>
<td>21.2±0.81</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FFA (mg/dl)</td>
<td>0.7±0.05</td>
<td>0.6±0.05</td>
<td>0.5±0.04</td>
<td>0.5±0.03</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>8.9±1.57</td>
<td>3.9±0.44</td>
<td>6.5±1.20</td>
<td>9.1±1.30</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.0±0.36</td>
<td>0.9±0.12</td>
<td>1.7±0.30</td>
<td>2.4±0.39</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HOMAβ</td>
<td>199.2±69.21</td>
<td>50.0±7.58</td>
<td>102.0±44.66</td>
<td>84.3±15.50</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>QUICKI</td>
<td>1.6±0.15</td>
<td>1.0±0.07</td>
<td>1.4±0.15</td>
<td>1.4±0.07</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.8±0.29</td>
<td>0.8±0.18</td>
<td>3.3±0.52</td>
<td>2.6±0.45</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>4733±291.51</td>
<td>5812±435.04</td>
<td>5213±312.69</td>
<td>5800±518.94</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>237.9±60.96</td>
<td>288.9±61.26</td>
<td>156.6±22.45</td>
<td>177.5±15.19</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>245.0±101.62</td>
<td>217.4±75.16</td>
<td>268.3±49.22</td>
<td>185.2±85.05</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>47.9±7.63</td>
<td>53.7±6.50</td>
<td>43.5±5.36</td>
<td>43.3±2.27</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Results are expressed by mean ± s.e.m. **Significant differences between groups of at least P<0.05. C, control; MS, maternal separation; HFS, high-fat sucrose; HFS-MS, high-fat-sucrose–maternal-separation; HDL, high-density lipoprotein; FFA, free fatty acids; HOMA, homeostasis model assessment; MCP-1, monocyte chemoattractant protein-1; ns, not significant.

DISCUSSION

In the current trial, we evaluated the peripheral effects of an HFS diet in adult female rats that had experienced an MS paradigm. Gender is one major variable that appears to confer differential vulnerability to stress. As Verbrugge (Verbrugge, 1985) and Frankenhaeuser et al. (Frankenhaeuser et al., 1976) reported, men and women differ in physiological and behavioral responses to stressors and in epidemiological patterns of stress-related illness. Despite the knowledge that women are more susceptible to men to stress-related mental illnesses such as major depression (Lewinsohn et al., 1998; Swaab and Hofman, 1995; Weinstock, 1999), many of the relevant studies in this field have been conducted in males, and less information is available on the response of female rodents to stressors. Sex differences in sensitivity to stress have also been documented in animals (Brown et al., 1996; Taylor et al., 2000) and there is no doubt that females are affected by some early environmental manipulations (Nunez et al., 1995). As expected, the intake of the hypercaloric diet induced changes in various obesity-related phenotypic and biochemical parameters (Milagro et al., 2006). Thus, increases in body weight and food intake in HFS-diet-fed groups were observed, together with higher fat pad mass and lower serum triglyceride, HDL and FFA levels. These results are in agreement with other studies, which have shown that the decrease in the lipid profile observed is characteristic of the rat models (Boque et al., 2009; Lomba et al., 2010). Moreover, serum leptin, an accurate obesity biomarker (Marti et al., 1999), was increased owing to the diet. Remarkably, the dietary treatment did not affect adiponectin, MCP-1 (one of the key factors involved in the initiation of obesity-related inflammation) (Melgarejo et al., 2009) or insulin resistance biomarkers. However, some studies (Galipeau et al., 2002; Horton et al., 1997) have reported that female rats are protected against the metabolic defects typically produced by high-carbohydrate feeding, which is also in agreement with previous work published by our group (Lomba et al., 2010).

In the present work, the nutriogenomic study involving the analysis of the expression of S2 genes in periovaric WAT and the subsequent validation highlighted some mechanisms related to diet-induced obesity. Interestingly, only two genes were significantly affected by the HFS diet: Ppargc1a and Lep. The small number of

Table 3. Differentially expressed genes in periovaric WAT

<table>
<thead>
<tr>
<th>Genes</th>
<th>Control (n=6)</th>
<th>MS (n=10)</th>
<th>HFS (n=7)</th>
<th>HFS-MS (n=11)</th>
<th>Diet</th>
<th>MS</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adpn</td>
<td>1.0±0.17</td>
<td>1.4±0.16</td>
<td>1.3±0.23</td>
<td>2.0±0.30</td>
<td>ns</td>
<td>&lt;0.05</td>
<td>ns</td>
</tr>
<tr>
<td>Lep</td>
<td>1.0±0.25</td>
<td>1.3±0.11</td>
<td>2.6±0.18</td>
<td>1.8±0.10</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>1.0±0.26</td>
<td>0.2±0.07</td>
<td>0.1±0.01</td>
<td>0.2±0.00</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Gene expression is shown in fold change in respect to the control group. Results are expressed by mean ± s.e.m. **Significant differences between groups of at least P<0.05. C, control; MS, maternal separation; HFS, high-fat sucrose; HFS-MS, high-fat-sucrose–maternal-separation; WAT, white adipose tissue; ns, not significant; ↑, increase of mRNA level; ↓, decrease of mRNA level. The full names of the genes are shown in supplementary material Table S2.
changed genes could be explained by the animals’ adaptation to the diet, as Fearnside et al. suggested (Fearnside et al., 2008).

As expected (Roberts et al., 2002), owing to the diet, Lept mRNA levels were increased in periovaric WAT, although no changes were observed in either Ccl2 or Adipoq mRNA levels. Pparγ1α, encoding a molecule involved in thermogenesis (Yudkin et al., 1999), was decreased by an HFS diet, which is in agreement with Arçarı et al. (Arçarı et al., 2009), who described that PGC-1α is downregulated by a high-fat diet.

In the scientific literature, there are contradictory results on the effect of a high-fat diet on the expression level of genes related to the metabolism of glucocorticoids in WAT. Thus, some studies have reported no changes in the levels of 11βHSD-1 or glucocorticoid receptor (GR) (Drake et al., 2005), some a decrease in 11βHSD-1 expression and activity (Livingstone et al., 2000) and another an increase (Walker, 2007). In this study, no changes were observed in any of the genes that we studied related to the metabolism of glucocorticoids. For blood corticosterone after a high-fat diet, the same conflicting outcomes have been reported, such as a lack of effect, as in this trial (Campion and Martinez, 2004; Maniam and Morris, 2010) or a decrease in the circulating levels of this hormone (Drake et al., 2005). Despite these differences, Maniam and Morris found that glucocorticoid response is blunted by the high-fat diet in rodents (Maniam and Morris, 2010), suggesting alterations in the hypothalamic-pituitary-adrenal (HPA) axis. Thus, Drake et al. have described that a high-fat diet reduces glucocorticoid concentrations, and that this is a key mechanism protecting against the metabolic complications of obesity (Drake et al., 2005).

The MS model in rats is considered a robust model of enhanced stress responsiveness (Aisa et al., 2007; Ladd et al., 2000; Levine, 2002; O’Mahony et al., 2009; Solas et al., 2010). This model has been associated with the development of anxiety-like behavior and learning impairments in adult rats. Moreover, different clinical and experimental studies have demonstrated that there is a clear relationship between the fetal environment and the risk of developing insulin resistance (Gluckman and Hanson, 2004). Furthermore, experiments in several animal species have shown an induction of insulin resistance and other manifestations of the metabolic syndrome by manipulating maternal nutrition or exposing the mother to synthetic glucocorticoids (Gluckman and Hanson, 2004). The MS paradigm decreased several fat pad masses and had a particularly remarkable effect in periovicar and subcutaneous WAT, suggesting that a neonatal adverse experience could lead to a different response to a hypercaloric diet intake in adulthood, compared with the control group. Curiously, no changes were found in food intake due to MS, indicating that the decrease in WAT weights could be due to previous modifications in peripheral tissues during lactation (De Moura and Passos, 2005). Thus, stress has been linked to obesity mainly through hypothalamic effects on food intake or peripherally through β-adrenergic (Troisi et al., 1991), glucocorticoid (Rosmond et al., 1998) or parasympathetic (Bartness et al., 2005) activity. As Kuo et al. stated (Kuo et al., 2007), in response to a stressor, some people lose weight, mainly via the β-adrenergic-mediated lipolytic pathway, whereas others gain weight, increasing the adipose tissue levels of cortisol, which could upregulate the expression level of neuropeptide Y (NPY) in the sympathetic nerves, stimulating the proliferation, differentiation and lipid filling of adipocytes. Moreover, the developmental plasticity, which is an adaptive process enabling an organism to respond to environmental insults acting in early life, could be reinforced by epigenetic mechanisms, such as DNA methylation and histone modifications (Cordero et al., 2011; Gluckman, 2011).

There is no consensus in the literature concerning the effect of neonatal manipulations on baseline corticosterone levels. Thus, some studies have found no differences (Kim et al., 2005; Ladd et al., 2004; Maniam and Morris, 2010), whereas others report decreased (Panagiotaropoulos et al., 2004; Papaioannou et al., 2002) or increased (Kawakami et al., 2007) corticosterone levels. In this sense, we did not observe any change in serum corticosterone levels; this lack of response could be attributed to the alteration by MS of the responsivity of the HPA axis to fasting (Kim et al., 2005). However, Aisa et al. reported a depressive-like behavior and increased axis responsiveness to acute stressors in female rats subjected to exactly the same MS paradigm (Aisa et al., 2008). Moreover, the behavioral test of males from the same litter of our rats demonstrated a hyperactivation of the HPA axis (Martisova et al., 2012), indicating that the rats of this trial might also have an altered HPA axis.

Regarding the effects of MS on the serum biochemical measures, all insulin resistance biomarkers were decreased owing to MS, but only in chow-fed rats, in accordance with previous studies (Delaunay et al., 1997; Lambillotte et al., 1997; Solas et al., 2010), which reported that glucocorticoids inhibit insulin secretion from pancreatic β-cells.

The nutrigenomic study showed a significant increase due to MS in Adpn, which is an adipose-specific transmembrane protein regulated by energy balance (Baulande et al., 2001) and which has been postulated to be part of the adipose-specific energy homeostasis sensor (Johansson et al., 2006), and a decrease in Pparγ1α (Carbone et al., 2012). Glucocorticoids are widely mentioned as triggering both lipolysis and adipogenesis, depending on the concentration, duration and type of glucocorticoid investigated, as well as the experimental model used (Kershaw et al., 2006; Masuzaki et al., 2001; Xu et al., 2009). Moreover, Yu et al. (Yu et al., 2010) and Campbell et al. (Campbell et al., 2011) reported that glucocorticoids could stimulate adipogenesis by acting on preadipocytes, and concomitantly could increase lipolysis through actions on mature adipocytes.

Estrogens might alter or interact with the HPA axis in regulating corticosterone release and in influencing cognitive function, and it has been suggested that estrogen in females might protect against the effects of corticosterone (Luine, 2002). However, in this study we did not observe any statistical change between the groups.

Our results suggest that early adverse events can lead to biological changes in the pups that persist into adulthood, inducing a different response to an HFS diet in later life involving alterations in the lipolysis and lipogenesis pathways. These changes could be explained via epigenetics, because DNA methylation and histone code could be modified during MS (Franklin et al., 2010) and these could be responsible for the different outcome induced by the HFS diet in this trial.

METHODS
Animals and experimental design
Timed-pregnant Wistar rats on gestation day 16, supplied by Charles River Laboratories (Barcelona, Spain), were individually...
housed in a temperature (21±1°C)- and humidity (55±5%)-controlled room on a 12-hour light-dark cycle with food and water freely available. As previously described (Aisa et al., 2007), on postnatal day (PND) 2, all pups were sexed and litters were randomly assigned to the control group (NH; n=3), for which pups were only briefly manipulated to change the bedding in their cages once weekly, or the separation group (S; n=4), for which pups were daily separated from their dam for 180 minutes from PND2 to 21 inclusive. Before manipulation of the MS pups, each dam was removed from her home cage and placed in an adjacent cage, then the pups were removed as complete litters, placed in an empty cage with standard bedding material and transferred to an incubator in an adjacent room. To compensate for the mother’s body heat, the temperature of the incubator was adjusted according to the age of the neonates: 32±0.5°C (PND2-5), 30±0.5°C (PND6-14) or 28±0.5°C (PND15-21). Rats were weaned on PND23 and only females were chosen for the present study. All subsequent experiments were performed when the rats were adults (60-75 days).

At 2 months of age, NH and S animals were randomized by weight into two dietary groups: standard chow diet (2014 Tekland Global 14% Protein Rodent Maintenance Diet, Harlan Iberica, Barcelona, Spain; C group, n=6 and MS group, n=10) and HFS diet [20% protein, 35% carbohydrate, of which 17% was sucrose, 45% fat, from Research Diets, Inc. (D12451); HFS, n=7 and HFS-MS group, n=11]. Animals were randomized to ensure that, in most cases, in each dietary treatment there were only three rats from each litter. Animals had ad libitum access to water and food during the experimental trial (35 days), and body weight was recorded three times a week. After animals were sacrificed by decapitation, blood and tissue samples were immediately collected and frozen (~80°C) for further analysis. All procedures were performed according to national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra.

Serum measurements
Circulating glucose was measured with an HK-CP kit (ABX Diagnostic, Montpellier, France) in automated COBAS MIRA equipment (Roche, Basel, Switzerland). Serum lepton (Linco Research, St Charles, MO), adiponectin (Linco Research, St Charles, MO), insulin (Mercodia AB, Uppsala, Sweden) and MCP-1 (Invitrogen, Carlsbad, CA) levels were determined by ELISA using automated TRITURUS equipment (Grifols International S.A., Barcelona, Spain). The HOMA, an index that estimates the insulin resistance based on the relationship between the fasting plasma insulin concentration and glucose concentration, was calculated as: [fasting plasma glucose (mM) × fasting serum insulin (µU/ml)]/22.5, as described elsewhere (Paternain et al., 2011). The homeostasis model of assessment of β-cell function (HOMAβ) was calculated as: ([fasting serum insulin (µU/ml) × 20]/[serum glucose (mM) − 3.5]) (Bianchi et al., 2010). The quantitative insulin sensitivity check index (QUICKI) was calculated as the inverse of the sum of the logarithms of the fasting insulin and fasting glucose (Cacho et al., 2008). Serum cortisolone level was determined using a commercially available enzyme immunoassay kit (IDS, Boldon, UK). Serum estradiol levels were analyzed on the Immulite 2000 analyzer by a competitive immunoassay using the reagents and calibrators supplied by the manufacturer (Diagnostic Products Corporation).

Real-time PCR
Total RNA was isolated from periocular WAT from the whole sample (n=35), according to Trizol manufacturer’s instructions (Invitrogen, Carlsbad, CA), followed by an additional purification step using the RNA easy kit (Qiagen, Germantown, MD). cDNA was synthesized using the RT2 First Strand Kit (Qiagen, Germantown, MD).

From the 35 animals included in the study, 20 (n=4 per experimental group) underwent analysis using a quantitative real-time PCR array (RT-PCR array) of 52 recognized genes related to obesity and glucocorticoid metabolism (supplementary material Table S2) following the manufacturer’s recommendations using the ABI PRISM 7900 HT Fast Real-Time PCR System (Applied Biosystems, Austin, TX). Obesity-related genes were analyzed with RT2 qPCR Primer Assay (Qiagen, Germantown, MD), whereas glucocorticoid metabolism genes were examined with Taqman probes for rats (Applied Biosystems, Austin, TX).

For the validation of the RT-PCR array, nine genes were selected and analyzed in the whole sample (n=35) using ABI PRISM 7900 HT Fast Real-Time PCR System (Applied Biosystems, Austin, TX) and Taqman probes for rats (Applied Biosystems, Austin, TX): Adpn (Rn 01502361_m1), Ccl2 (Rn 00580555_m1), Cd36 (Rn 00580728_m1), Lep (Rn 565158_m1), Lipe (Rn 00563444_m1), Mgl1...
Gene expression levels were always normalized using GAPDH mRNA as an internal control. Fold-change between the groups was calculated using the 2^ΔΔCt method (Paternain et al., 2011).

**Statistical analysis**

All results are expressed as mean ± standard error of the mean (s.e.m.). Data and interactions were evaluated by two-way factorial ANOVA (Diet, MS, Interaction) followed by Bonferroni test for multiple comparisons. Repeated-measures ANOVA was applied for analysis of food intake. The level of probability was set at P<0.05 as statistically significant. All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL).

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**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.009043/-/DC1

**REFERENCES**

Aisa, B., Tordera, R., Lasheras, B., Del Rio, J. and Ramirez, M. J. (2009). Maternal separation on hypothalamic-pituitary-adrenal responses, cognition and food intake. The level of probability was set at P<0.05 as statistically significant. All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL).


